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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Wold et al.	**Group No.:**	1632
Serial No.:	09/351,778	**Atty. Docket No.:**	66153-7775
Filed:	07/12/1999		
For:	Replication-Competent Anti-Cancer Vectors	**Examiner:**	Priebe, Scott David

DECLARATION, PURSUANT TO 37 C.F.R. §1.131, OF WILLIAM S.M. WOLD, Ph.D., ANN E. TOLLEFSON, Ph.D., KONSTANTIN DORONIN, Ph.D. AND KAROLY TOTH, D.V.M.

We, William S.M. Wold, Ph.D., Ann E. Tollefson, Ph.D., Konstantin Doronin, Ph.D. and Karoly Toth, D.V.M., declare and state as follows:

1. All of the statements made herein of our own knowledge are true and all statements made on information and belief are believed to be true.

2. We are co-inventors of the inventions described and claimed in the above referenced patent application, filed July 12, 1999.

3. We conceived of a recombinant adenovirus vector which is replication-competent in neoplastic cells and which overexpresses an adenovirus death protein prior to March 3, 1997. We worked diligently, continuously and uninterrupted from prior to March 3, 1997, until we reduced the recombinant adenovirus to practice. The information contained in the documents of the attached exhibits was contemporaneously recorded on the dates indicated therein. Further, those exhibits are

true copies of the originals except to the extent that certain sensitive dates have been redacted. All noted dates that have not been redacted are accurate.

4. Claim 1 of the '778 application is directed to a recombinant adenovirus vector that is replication-competent in neoplastic cells and that overexpresses adenovirus death protein (ADP).

5. On or before March 3, 1997, we conceived of a recombinant adenovirus vector that is replication-competent in neoplastic cells and that overexpresses ADP, as evidenced by the activities set forth below. Unless otherwise noted, all of the documents referred to in the bullet point sections below are dated prior to March 3, 1997:

• Our early work focused on the E3 transcription unit (also referred to as the E3 region) of Adenovirus (Ad), and understanding the function of the various proteins encoded by that region. We observed that Ad comprising an ADP (11.6K) mutation (i.e. a mutation in the gene coding for ADP) developed plaques slower, and that those plaques were smaller, than Ad with wild type levels of ADP expression (see Exhibit A, page1 [A1], where dl712 is an ADP mutant and Ad5 is a wild-type adenovirus).

• In addition, we observed that some mutant Ads that have portions of the E3 region deleted, other than the gene for ADP, produced larger plaques than the wild-type Ad. See page 2 of Exhibit A (page A2), which is a notebook page memorializing these observations. This page shows that dl753 produced larger plaques than wild-type.

• Plaque development assays indicated that ADP mutant Ad produced plaques at different rates than wild type Ad (see pages A3-A17, which are plaque assay results which show that dl753 and dl732 produce plaques faster than wild type Ad, and ADP deletion mutants form plaques slower than wild-type). Note that "rec700" represents wild-type Ad, and "dl 753" and "dl 732" are adenoviruses that have deletions in the E3 region [not in the open reading frame for ADP] that result in increased synthesis of ADP; and dl712, dl801, dl742, dl708 and dl7001 have deletions in the coding region for ADP that preclude synthesis of functional ADP.

• Gel electrophoresis studies were carried out which indicated that E3 deletion Ad produced higher levels of ADP (see Exhibit A, pages A18-A20, which are photocopies of those gels that show that dl753 (an E3 deletion mutant) produces more ADP than rec700 (which produces wild-type levels of ADP).

• Immunofluorescence assays were carried out that confirmed these findings that E3 deletion mutants produce more ADP than wild-type (See Exhibit A, pages A21-A25, where dl732 and dl753 are E3 deletion mutants that overexpress ADP, dl712 is an E3 mutant in which the gene for ADP is deleted, and rec700 is wild-type).

• In virus release assays, we observed that the ADP mutants that had large plaques released more virus than both wild-type Ad and ADP mutants that had small plaques (A26).

• This early work led us to hypothesize that ADP is required for cell lysis, and release of Ad . We also hypothesized that over-expression of ADP may cause cell lysis more rapidly, and therefore, that vectors that overexpress ADP may be useful as anti-cancer agents.

• A proposal, entitled "Adenovirus E3-11.6K Protein as a Cell Death-Promoting Agent," was sent to Dr. Rae Lyn Burke of Chiron Corp. That Proposal is submitted herewith as Exhibit B. At page 7 of that proposal, the concept of overexpressing ADP (11.6K) is discussed. Further, at page 4 the KD/GZ class of adenovirus vectors are described, where it is stated that "[t]he nondefective vectors generally have the E3 transcription unit deleted and replaced with the transgene" [e.g., ADP]. The concept that deletion of the E3 transcription unit and insertion of the ADP gene would result in overexpression is based on the understanding that deletion of most or all of the E3 genes other than the ADP gene facilitates overexpression of ADP mRNA by reducing competition for splicing of the major late pre-mRNAs. That concept is put forth in the instant specification at page 15, lines 12-17. That concept is also discussed in the proposal at page 7, where it is stated that "...we will determine whether 11.6K overexpressed during early stages of infection can promote cell death. The 11.6K gene will be built into our mutant, dl7001, which lacks the entire E3 region but expresses all other adenovirus genes... Cells will be infected with the dl7001-11.6K vector...and cell death will be monitored."

6. Prior to March 3, 1997, we constructed a recombinant adenovirus vector that is replication-competent in neoplastic cells and that overexpresses ADP. Specifically, KD1 is a recombinant adenovirus vector that is replication-competent in neoplastic cells and that overexpresses ADP. KD1 was constructed by cotransfecting dl1101/1107 DNA that had been digested with EcoRI and plasmid p54 DNA comprising the ADP gene into 293 cells. DNA from resulting plaques was confirmed as KD1 by PCR. The following activities, which occurred prior to March 3, 1997 are related to the construction and evaluation of ADP overexpressing vectors generally, and specifically KD1:

• High titer dl1101/1107 stocks were expanded for the purpose of examining whether this virus with a mutation in the E1A gene would grow well on cancer cell lines and not on non-cancerous cell lines, and also for the purpose of deleting the E3 region of an Ad strain and inserting the ADP from Ad5. (See Exhibit D1) (dl1101/1107 serves as the "backbone" of the KD strains described in the 09/351,778 application, see page 22, Table 1).

• High titer dl1101/1107 stocks were confirmed. (D2-D3).

• The EcoRI-A fragment of Ad5 containing the DNA sequence encoding ADP was purified. (D4)

• Various deletions and insertions were made in the backbone adenoviral E3 region by PCR. For example, in the construction of KD1, the ADP gene was isolated from the Ad5 genome by PCR using the primers named KD6 and KD7 that added PacI restriction sites to the DNA fragment containing the ADP gene. (E3, E4 and E9). This ADP-containing PCR fragment was cloned into a PacI site in the plasmid named pL2 to produce the shuttle plasmid named p54 (F7-F8) (D9). Another name for plasmid pL2 is pLKHE2A + Bam → end + Cla. The sequences for primers KD6 and KD7 are presented in Appendix E1, E3, E4 and E9 and are described in the examples in the '778 application. These primers as well as other primers were purchased from Gibco BRL. Exhibit E comprises order sheets and certificates of analysis for a number of those primers, along with contemporaneous notes that describe the primers. (E1-E10)

• Growth curves to assess whether dl1101/1107 is attenuated for growth as compared to dl309 in HEL299 cells. (I1-I31)

• Various plasmids used to construct the KD vectors were sequenced. (F1-F8)

• The DNA sequence of the ADP gene inserted into plasmid p54 was determined using the KD6 and KD7 primers (F7-F8)

• The ADP gene was successfully subcloned into the shuttle plasmid vector, culminating in the isolation of the KD1 shuttle plasmid vector. (Exhibit F5-F6) (D9)

• dl1101/1107 DNA that had been digested with EcoRI was cotransfected into 293 cells together with plasmid p54 DNA. (D39)

• Seven putative KD1 plaques from the above transfection experiment were observed. (C1) (KD) (WW) DNA from these plaques is confirmed as KD1. (C6)

• New DNA was prepared from cells infected separately with viruses isolated from ten plaques that resulted from the above co-transfection experiment. (C6)

• The above DNA was analyzed by restriction enzyme digestion and PCR. Three of the plaques were shown to contain the E3 region from plasmid p54; that is, three of the plaque isolates were KD1. (C9) (D48-49)

• A second cotransfection experiment was performed using EcoRI-digested dl1101/1107 DNA plus plasmid p54 in a repeat attempt to construct KD1. (D47). On 3/6/97 the Hirt supernatant DNA from four plaque isolates had been analyzed by PCR;

three of the plaque isolated had the E3 region of KD1. (D52) (C16). Thus, prior to March 3, 1997, KD1 had been constructed in two separate experiments.

7. From the time the KD1 vector was made, we continued to work on construction and characterization of additional replication competent vectors that overexpress ADP, as evidenced by the activities listed below. This work eventually led to the KD3, GZ1 and GZ3 vectors described in the specification of the '778 patent, particularly in the Examples. Our experiments show that KD1 overexpresses ADP. Specifically, on or about May 20, 1997, we show that KD1 develops plaques faster than dl309, which expresses wild-type levels of ADP. Faster plaque development is correlated with overexpression of ADP. Based on the plaque development assay, we concluded that KD1 overexpresses ADP. In addition to the activities listed below, certain activities occurred on a continual basis. Those activities include, among others, maintenance of stocks of human cell lines used in evaluating the vectors; maintenance of bacterial cell cultures used to generate stocks of the various plasmids used to construct the claimed vectors; experiments such as virus growth curves, virus plaque assays, virus spread assays, virus induced cell death assays, all of which require multiple days to complete; lab meetings and informal discussions of progress and strategies for future experiments. The following activities are evidence of our continuous efforts:

3/3/97-3/7/97:

- From prior to March 3, 1997 through at least March 7, 1997, plaque assays of KD1 (also referred to as 544 in the attached exhibits) continued to progress and observations were made. Also, stocks of cells comprising the recombinant virus with the E3 region deleted and ADP inserted were maintained. (C15-16)

- On 3/6/97 the Hirt supernatant DNA from four plaque isolates from the second cotransfection experiment performed using EcoRI-digested dl1101/1107 DNA plus plasmid p54 had been analyzed by PCR; three of the plaque isolated had the E3 region of KD1. (D52) (C16) .

- Growth curves with dl1101/1107 and dl309 on HEL299 cells or WI38 cells continue. (I32)

3/7/97-3/14/97:

- Plaque assays of the KD1 vector continued. (C19)

- Virus growth assays continued on WI38 cells to assess the growth of dl1101/1107. (C20)

- Assays continue to assess dl1101/1107 and dl309 in growth arrested vs. growing HEL299 cells. (C21, I33-34)

- Initial stocks of the KD1 virus had been prepared. (C19)

3/14/97-3/21/97:

- The E4 promoter region of dl1101/1107 was sequenced to help in constructing an E4 promoter substituted virus. (C22)

- Virus growth assays continued on WI38 cells to assess the growth of dl1101/1107. (C23)

- Sequencing of plasmids used to construct the KD vectors continued using various primers. (F9-F12)

- Assays continued to assess dl1101/1107 and dl309 in growth arrested vs. growing HEL299cells. (C24-25)

3/21/97-4/4/97:

- HEL299 growing and growth arrested cells are infected with dl1101/1107 and dl309 and virus growth assays are in progress. (C26, I35-36)

- dl1101/1107 extracted from WI38 permissive cells is titered. (C27)

3/21/97-4/11/97:

- dl1101/1107/EcoRI and p101 or p111 are cotransfected and cell cultures observed to identify plaques that may comprise the KD2 and KD3 vectors. (C28)

4/4/97-4/11/97:

- Growth assays with dl1101/1107 and dl309 continue in HEL299 cells. (C29)

- Titer experiments continue with dl1101/1107 extracted from WI38 cells. (C30)

4/11/97-4/18/97:

• Plaques with putative KD2 and KD3 virus are observed, and Hirt preps of DNA from those plaques are made. (C31)

• Titer experiments continue with dl1101/1107 extracted from WI38 cells, and preliminary data are collected. (C32)

• Growth curve experiments with dl1101/1107 and dl309 on growth arrested and growing HEL299 cells are continued and data are compiled. (C34-35)

4/18/97-4/25/97:

• PCR and restriction enzyme digestions are done to confirm KD2, and Hirt DNA preps are made for putative KD3 plaques. (C36)

• Titer experiments continued with dl309 and dl1101/1107 extracted from infected WI38 cells. (C37-39)

• Growth curve experiment was started with dl1101/1107 and dl309 viruses on MCF-7 human breast cancer cells. (C37)

• HEL299 cells infected with dl1101/1107 and dl309 are frozen for growth curve analysis. (C40)

• Sequencing of plasmids used to construct KD vectors continued using various primers. (F13-F17)

• Cell culture supernatant stock of KD virus is given to lab technician (SB) for preparation of a high titer CsCl-banded stock. (C36)

4/18/97-5/2/97:

• Titrations of virus extracted from HEL299 cells infected with dl1101/1107 and dl309 continues. (C45)

• Plaque development assay started with KD1 comparing it to dl309 (wild-type E1A, normal ADP expression), dl1101/1107 (E1A mutation, wild-type ADP expression), pm734.1 (wild-type E1A, no ADP expression), and dl7001 (wild-type E1A, no ADP gene). The cell lines used were 293 and A549 cells. (C41) (D90-D92) . The results of this plaque assay are shown graphically at C56. The KD1 and dl309 plaques developed approximately 2 to 3 days faster than did the plaques of dl1101/1107. The fact that KD1 plaques developed more rapidly than those of dl1101/1107 indicates that KD1 overexpresses ADP.

• SB (lab technician) obtained the KD1 virus from KD and began experiments to expand the amount of virus. (K21)

4/25/97-5/2/97:

• KD3 vector (referred to as dlE3Xba + ADP) was confirmed by PCR and restriction enzyme digestion. (C41)

• Experiments to expand KD1 continue. (K22 K23)

5/2/97-5/9/97:

• Stocks of dlE3 + ADP were grown and monitored for plaque development. Also, cells transfected with KD1, KD2 and KD3 are monitored for development of plaques relative to dl309. (C47, C49)

• Growing and growth arrested dl1101/1107 infected HEL299 cell extracts are titered for growth curve. (C50)

• Expansion of KD1 continues. (K24) (C49)

5/9/97-5/23/97:

• Plaque development assays continue, to asses KD1, KD2 and KD3 relative to wild-type. (C51)

• Plaque assays to assess expression of ADP by KD1 relative to several viruses with wild-type E1A genes continue. Fast plaque formation by KD1 indicates ADP overexpression. (H2-H3)

• Growth curves with dl309 and dl1101/1107 extracted from HEL299 cells continue, and data is collected at various intervals. (C52-53)

• Sequencing of primers used to construct KD vectors continued. (F18-F19)

• Titering of dl309 DNA extracted from 293 cells is initiated. (I37)

• Growth curves with dl309 and dl1101/1107 extracted from HEL299 cells continue. (I38)

• Expansion of KD1 continues. (K25) (AT)

5/13/97-6/2/97:

• Plaque assay to assess KD1. Results indicate that KD1 overexpresses ADP. (K25-K28)

• Expansion of KD1 continues. (K26)

5/20/97-6/8/97:

• Plaque assays of various viruses including KD1 and dl751. (H4-H10). The plaque development curve indicates that KD1 formed plaques as well as or better than dl751, a virus that overexpresses ADP. This suggests that the virus had good ADP expression and function. (K27-28) (H4-H9). KD1 was plaque assayed along with dl751 (which expresses higher than normal quantities of ADP), two preparations of dl707 (a mutant in which a portion of ADP is deleted and which therefore would be expected to have delayed kinetics of plaque formation), and pm734.9 (an ADP mutant with a three amino acid mutation at the C-terminus of ADP [listed as PME on the plaque assay counts]).

5/23/97-5/30/97:

• Plaque development assays were monitored and results collected. C56 is a graphical presentation of results indicating that KD1 develops plaques as quickly as dl309 and quicker than dl1101/1107, and that KD2 and KD3 develop plaques more quickly than dl1101/1107. (C55-C58)

5/30/97-6/27/97:

• Efforts focused on further characterization of the KD vectors, and improvements on those vectors, such as for example substituting the E4 promoter. Growth curves with dl1101/1107 continued, and Plaque development assays with KD1 indicated that KD1 develops plaques somewhat slower than dl309. (C65-C82)

• Infections were done for immunofluorescence staining for ADP. Viruses used for infection were dl309, KD1, KD2 and KD3. It is noted that KD shows much more staining for ADP than the other viruses (both at 27 and 49 hours post-infection). This is supporting data for the overexpression of ADP by the KD viruses. (H1-H2)

8. As evidenced by the foregoing, we confirmed that KD1 overexpresses ADP by immunofluorescence assays utilizing an ADP-specific antibody on or about June 23, 1997.

9. We confirmed that KD1 overexpresses ADP by Western blot on or about December 1, 1997. (Exhibit L, pages L2-L4)

10. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. 1001) and may jeopardize the validity of the application or any patent issuing thereon.

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